REMARKS

Claims 18-21 are added as new claims. Claims 18-21 each depends from independent claim 12 and are directed to preferred embodiments of the recited method using specific compounds. Support for new claims 18-21 can be found, for example, at pages 41, 42 and 47-48 of the original specification. Accordingly, no new matter is presented.

1. Response to Claim Rejections Under 35 U.S.C. § 103

A. Bell or Brooker et al in view of Feldner et al and/or Foucault et al

Claims 12 and 13 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over either of Bell (U.S. Patent No. 4,551,413) or Brooker et al (U.S. Patent No. 3,715,351), in view of Soini et al (U.S. Patent No. 5,891,738) combined with Feldner et al ("Non-linear optical properties of specific polymethines: influence of substituents and chain length", MCLC S&T Sec. B, Nonlinear Optics vol. 26(1-3), pp. 99-106 (2000)) and/or Foucault et al ("Two photon absorption in organic dyes relation with the symmetry of levels", Optics Comm. Vol. 15(3), pp. 412-415 (1975)).

The references are applied as in the previous Action dated April 5, 2006. In response to the Amendment filed on August 18, 2006 and the arguments presented therein, the Examiner states that Applicants did not address the primary references now cited in the present rejection.

Applicants respectfully traverse the rejection.

First, Applicants note that in the paragraph bridging pages 3-4 of the Action, the Examiner mentions Kitagiri et al, which is believed to be an error since Kitagiri et al was not included in the rejection and since Kitagiri et al was clearly distinguished in the Amendment filed on August 18, 2006. Applicants respectfully request clarification from the Examiner on this point in the next Action for the record.

Further, Applicants note that it was previously argued that none of the cited references disclose a method for inducing a non-resonant two-photon absorption comprising irradiating with laser beams having a wavelength longer than the linear absorption band of the presently claimed compound, or a method for generating an emission of non-resonant two-photon by inducing non-resonant two-photon absorption and generating an emission as recited in the present claims. Thus, Applicants previously addressed all references cited in the previous Action.

Specifically, Bell and Brooker et al are related to dyes and do not disclose, suggest or mention a method for inducing a non-resonant two-photon absorption or a method for generating an emission of non-resonant two-photon by inducing non-resonant two-photon absorption and generating an emission as recited in the present claims.

Further, there is no motivation or suggestion to combine Bell or Brooker with Soini et al, Feldner et al and/or Foucault et al.

Bell is directed to a recording element for optical data storage which comprises specific dyes and Brooker et al is directed to cyanine dyes used as spectral sensitizers for photographic silver halide emulsions.

Soini et al is directed to a biospecific multiparameter assay method, which is non-analogous art. The Example 8 of Soini et al (US 5,891,738) discloses an example in which a dye is excited by using exciting light (578 to 772 nm) corresponding to the linear absorption wavelength of BHDMAP. Table 1 of Lee et al (copy attached), indicates that the absorption maximum of BHDMAP is 786 nm.

Because the dye in Soini et al is excited by exciting light (578 to 772 nm) having a wavelength shorter than the wavelength of the dye, Soini et al is entirely different from "inducing a non-resonant two-photon absorption comprising irradiating with laser beams having a wavelength longer than the linear absorption band of the compound," as recited in claims 12 and 13 of the present application.

Feldner et al and Foucault et al relate to studies of the nonlinear optical properties of specific dyes but do not mention, teach or suggest a method for inducing a non-resonant two-photon absorption by irradiating the compound of formula (1) or formula (3) or a method for generating an emission of non-resonant two-photon by irradiating the compound of formula (1) or formula (3) and inducing non-resonant two-photon absorption and generating an emission as recited in the present claims. Thus, one of ordinary skill in the art would not have been motivated to combine the references as suggested by the Examiner since there is no objective reason taught in the references for modifying the references. It has been established that the prior art must suggest the desirability to modify or combine disclosures. The fact that references can be combined or modified is not sufficient to establish *prima facie* obviousness. *In re Mills,* 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990).

B. Nakamura et al or JP '466 in view of Devoe et al, Feldner and Foucault et al

Claim 12 is rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over either of Nakamura et al (U.S. Patent Appln. No. 2002/0045128) or JP 2002-072466, in view of Devoe et al (WO 01/96917), Feldner et al and Foucault et al.

Applicants respectfully traverse the rejection and submit that the cited references do not teach or suggest the present invention, whether taken alone or in combination, based on the following.

Nakamura et al specifically teaches that the recording material disclosed therein is for "heat-mode exposure". Paragraph [0013]. Further in paragraphs [0014] and [0015], Nakamura et al discusses the disadvantages of photon-mode exposure and at paragraph [0183] it is specifically stated that substantially no image is formed through photon-mode exposure and that the recording material of Nakamura et al requires heat-mode exposure for image formation. Therefore, Nakamura et al teaches away from photon-mode exposure. Thus, one of ordinary skill in the art would not have been motivated to combine Nakamura et al with Devoe et al, Feldner et al and/or Foucault et al with a reasonable expectation of success.

Further, JP '466, Devoe et al, Feldner et al and/or Foucault et al are not related. JP '466 relates to a photopolymerizable composition suitable for lithographic printing, whereas Devoe et al is related to methods of fabricating encapsulated optical elements using multi-photon polymerization techniques, and Feldner et al and Foucault et al merely describe nonlinear optical properties of specific dyes. However, there is no specific teaching or objective reason for combining the references as suggested by the Examiner to arrive at a method for inducing a non-resonant two-photon absorption by irradiating the compound of formula (1) or formula (3) as presently claimed.

C. Urano et al or JP '009 in view of Marder et al, Devoe et al, Feldner et al and Foucault et al

Claim 12 is rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Urano et al (U.S. Patent No. 6,153,356) or JP 09-134009 in view of Marder et al (WO 02/079691), Devoe at al, Feldner et al and Foucault et al.

Applicants respectfully traverse the rejection and submit that the cited references do not teach or suggest the present invention, whether taken alone or in combination.

First, Applicants note that on page 6 of the Action, the Examiner refers to Nakamura et al and JP 2002-072466, which are not included in the rejection. Therefore, Applicants are not certain of the Examiner's basis for asserting that the combination of Urano et al or JP '009 in view of Marder et al, Devoe et al, Feldner et al and Foucault et al renders the presently claimed invention obvious.

Neither of Urano et al and JP '009 teaches or suggests the presently claimed method and there is no objective reason to combine the references as suggested by the Examiner. Specifically, Urano et al and JP '009 relate to photopolyermizable compositions suitable for lithographic printing, whereas Devoe et al is related to methods of fabricating encapsulated optical elements using multi-photon polymerization techniques, and Feldner et al and Foucault et al merely describe nonlinear optical properties of specific dyes. As discussed above, there is no specific teaching or objective reason for combining the references as suggested by the Examiner to arrive at a method for inducing a non-resonant two-photon absorption by irradiating the compound of formula (1) or formula (3) as presently claimed.

In summary, Applicants respectfully submit that the Examiner has not made a *prima facie* showing of obviousness. The present invention is patentable over the cited references. Accordingly, Applicants respectfully request withdrawal of the §103 rejections.

II. New Claims 18-21

New claims 18-21 are directed to preferred embodiments of the present invention using specific compounds. New claims 18-21 depend from independent claim 12 and are patentable for at least the same reasons.

III. Conclusion

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

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Cytometry 21:120–128 (1995)

Near-IR Dyes in Three-Color Volumetric Capillary Cytometry: Cell Analysis With 633- and 785-nm Laser Excitation

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Several fluorescent dyes that absorb in the near-infrared are described. The photostability and aggregation properties of the dyes were examined. Two of the dyes, BHMP and BHDMAP, emit at 805 nm and were useful dyes for protein labeling. A dual-laser, three-color scanning instrument was constructed. CD3+CD4+ and CD3+CD8+ populations were enumerated in undiluted, whole blood based on the fluorescence of Cy5, Cy5.5 and BHMP. © 1995 Wiley-Liss, Inc.

Key terms: Diode laser, near-IR fluorescent dyes, CD4 enumeration

The detection of fluorescently labeled biological probes has become integrated into many research tools, encompassing instrumentation and reagents in flow cytometry (21, 9), oligosaccharide sequencing (10), fluorescence microscopy (3) and DNA mapping (8) and sequencing (23). The requirements for fluorescent probes for biological labeling include water-solubility, high extinction coefficients and quantum yields, and photostability. For multiparameter analysis the availability of spectrally resolvable dyes is also required. Fluorescent probes span the UV and visible wavelengths (300–800 nm) (6). The development of fluorescent probes has recently progressed towards longer wavelengths (600–800 nm) in order to utilize inexpensive light sources and to allow excitation in a region of the spectrum that has less absorption from biological sources.

We wished to exploit the low cost, compact size and high reliability of He-Ne and diode lasers for fluorescence detection of labeled cells. A cell-counting device, the Volumetric Capillary Cytometer (VCC), based on the 633-nm He-Ne laser has been previously described (11). In this paper, we describe the addition of a diode laser to the VCC that permits two orthogonal channels at low additional cost. To our knowledge, this system is the first demonstration of cell enumeration using dyes emitting in the near infrared (IR) spectral region.

Diode lasers at 635, 670 and 780 nm are commercially available. The arylsulfonate cyanine dyes, Cy5 and Cy5.5, are excellent fluorophores for the diode lasers at 635 and 670 nm (14, 24). Squaraine dyes have also been developed that absorb at 635 nm (22). Dyes that can be excited at 780 nm have been described for covalent labeling of proteins (19, 20) and oligonucleotides (13). Detection of oligonucleotides with these dyes appeared to be effective; however, detection of proteins labeled with these dyes apparently required solution in 50% methanol, presumably to decrease dye aggregation.

A major challenge in designing dyes for protein labeling is minimizing aggregation of the dye, since aggregated dye is essentially non-fluorescent. Most synthetic organic dyes are large, planar structures that are intrinsically hydrophobic and have a tendency to aggregate in aqueous solutions. Dyes that absorb in the red end of the visible spectrum (600–800 nm) have additional tendency to stack due to their increased size and hydrophobicity. Aggregation is enhanced in high salt solutions and when dyes are in high local concentrations on protein surfaces. The absorbance maxima of the aggregated dye in both cases is shifted to shorter wavelengths compared to the monomeric form. The effect of high salt on dye aggregation is similar to the increase in stacking interactions, and hence, T_m, of double-stranded DNA in high ionic strength solutions (17).

Cyanine dyes are well-known for their tendency to aggregate, particularly in aqueous solution. Dye aggregation can be minimized by constructing dyes that are highly ionic. The structures of Cy5 and Cy5.5 include aromatic sulfonate groups; these dyes have minimal aggregation in 3.8 M NaCl or on proteins. Presumably, the negatively charged sulfonate groups both increase water solubility and sterically prevent stacking. A similar effect could be expected from a positively charged group attached to the dye chromophore.

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We describe here heptamethine cyanine dyes containing pyridinium ions at the central methine. These dyes were inspired by the work of Strekowski et al., in which the central methine group of heptamethine cyanine dyes were substituted with various neutral aromatic moieties (19, 20). We discovered that by substituting dialkylaminopyridinium ions the resulting dicationic dyes are extremely water-soluble and surprisingly stable. The dyes absorb in the near IR, exhibit minimal aggregation and are suitable for protein labeling and cell identification.

MATERIALS AND METHODS

Chemicals

4,5-Benzo-2,3,3-trimethylindoline and IR125 was purchased from Fisher Scientific. Triethylammonium acetate (2 *M*) was purchased from Applied Biosystems (Foster City, CA). Reverse-phase thin-layer chromatography plates were purchased from Analtech (Newark, DE). Succinimidyl esters of Cy5.29 and Cy5.5 were purchased from Biological Detection Systems (Pittsburgh, PA). Monoclonal antibodies were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). Sephadex G-25 was purchased from Pharmacia Biotech (Piscataway, NJ). All other chemicals and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Analytical Methods

Proton NMR spectra were performed by Acorn NMR (Fremont, CA) using a Nicolet 360 MHz NMR spectrometer. UV-visible spectra were obtained on a Beckman DU 650 spectrophotometer. Fluorescence spectra were obtained on a SLM Aminco AB Series 2 Luminescence Spectrometer equipped with a Hitachi 2658 photomultiplier. Analytical HPLC was performed on Waters 600E system controller with a Waters 996 photodiode array detector. Separation systems utilized reversed-phase columns (Poros R2/H, 4.6 × 100 mm or Waters Nova-Pak C8, 3.9 × 150 mm) and gradients of 0.1 M triethylammonium acetate and acetonitrile.

Optics and Mechanics

Helium-neon lasers (3 mW, 1103P) were purchased from Uniphase Corp (San Jose, CA). Diode lasers (50 mW, HL7851G) were purchased from Hitachi (Brisbane, CA). Filters were purchased from Omega Optical (Brattleboro, VT). Photomultiplier tubes (3896) were purchased from Hamamatsu (Bridgewater, NJ). Galvanometers were purchased from General Scanning (Watertown, MA). Glass capillaries (35 × 500 μM) were purchased from Vitro Dynamics (Rockaway Beach, NJ). Software analysis was performed with Labview v.3.0 from National Instruments (Austin, TX).

1-(5′-Carboxypentyl)-2,3,3-trimethylindolinium bromide

A mixture of 1,3,3-trimethylindoline (3.8 g, 23 mmol) and bromohexanoic acid (4.6 g, 23 mmol) was stirred under nitrogen at 110°C for 12 h. The red solid was triturated with refluxing ethyl acetate (2 × 20 mL) followed by refluxing acetone (20 mL). The pink powder was filtered and air dried. Yield: 6.3 g, 18 mmol, 77%. ^{1}H NMR (CD$_3$OD) δ 1.54 (m, 2H), 1.61 (s, 6H), 1.71 (m, 2H), 2.00 (m, 2H), 2.34 (t, J = 7.3 Hz, 2H), 4.53 (t, J = 7.7 Hz, 2H), 7.62–7.9 (m, 4H). The 2-methyl protons are acidic and exchanged with the deuterated solvent.

N-[5-Anilino-3-chloro-2,4-(propane-1′,3′-diyl)-2,4-pentadien-1-ylidene]anilinium chloride

A modification of the procedure of Reynolds and Drexhage was used (16). A solution of dimethylformamide (7.6 g, 100 mmol) and dichloromethane (2 mL) was cooled in ice with stirring under nitrogen. Phosphorus oxychloride (12 g, 75 mmol) in dichloromethane (2 mL) was added dropwise over 10 min. Cyclohexanone (2 g, 20 mmol) in dichloromethane (3 mL) was added dropwise over 10 min. The solution turned yellow. The solution was refluxed for 3 h and became orange.

The solution was poured onto 50 g of ice. The organic layer was separated and discarded. Aniline (5 g, 54 mmol) was added. A dark purple precipitate formed immediately. The solid was filtered and washed with 1 N HCl and air dried. Yield: 1.9 g, 5.3 mmol, 27%.

2-[4′-Chloro-7′-(1″-[5‴-carboxypentyl]-3″,3″-dimethylindolin-2″-ylidene)-3′,5′-(propane-1⁗,3⁗-diyl)-1′,3′,5′-Heptatrien-1′-yl]-1-(5′′′′′-carboxypentyl)-3,3-dimethylindolinium bromide (BHCl)

A solution of 1-(5′-carboxypentyl)-2,3,3-trimethylindolinium bromide (1.2 g, 3.4 mmol), *N*-[5-anilino-3-chloro-2,4-(propane-1′,3′-diyl)-2,4-pentadien-1-ylidene-]anilinium chloride (0.57 g, 1.6 mmol), sodium acetate (0.28 g, 3.4 mmol) and ethanol (20 mL) was refluxed for 30 min. The solution was concentrated to dryness and the residue was washed with 1:1 ethyl acetate:hexane (20 mL). The residue was dissolved in methylene chloride (50 mL) and washed with 1 N HCl (2 × 20 mL), brine (20 mL) and dried (Na$_2$SO$_4$). The sticky solid was dissolved in methylene chloride (2 mL) and precipitated with ethyl acetate (50 mL). Residual solvent was removed to provide a purple solid (0.65 g, 0.85 mmol, 53%). ^{1}H NMR (CD$_3$OD) δ 1.51 (m, 4H), 1.7 (m 4H), 1.74 (s, 12H), 1.87 (m, 4H), 1.97 (m, 2H), 2.35 (m, 4H), 2.75 (m, 4H), 4.18 (m, 4H), 7.32 (m, 2H), 7.4–7.55 (m, 8H), 8.45 (m, 2H).

BHDMAP

To a solution of BHCl (50 mg, 0.066 mmol) in DMF (1 mL) was added 4-dimethylaminopyridine (20 mg, 0.16 mmol). The reaction was monitored by thin-layer chromatography (TLC) on reverse-phase plates with methanol elution. The starting chloride had an Rf of 0.25 and the product had an Rf of zero. After 15 h at ambient temperature, the reaction was complete. Ether (5 mL) was added, and the dark precipitate was separated by centrifugation. The solid was redissolved in DMF (0.5

mL) and precipitated with ether (5 mL). The green, gold-reflecting solid was dried on a rotary evaporator. Yield: 45 mg, 0.051 mmol, 77%. ^1H NMR(CD_3OD) δ 1.41 (s, 12H), 1.48 (m, 4H), 1.69 (m, 4H), 1.85 (m, 4H), 2.11 (m, 2H), 2.36 (m, 4H), 2.84 (m, 4H), 3.49 (s, 6H), 4.21(m, 4H), 6.42 (d, J = 11.8 Hz, 2H), 6.94 (d, J = 11.8 Hz, 2H), 7.3–7.5 (m, 10H), 8.30 (d, J = 6.7 Hz, 2H).

BHMP

BHMP was synthesized from BHCl (50 mg, 0.066 mmol) and 4-morpholinopyridine (15) (20 mg, 0.12 mmol) by the same procedure as BHDMAP. Yield of green solid: 48 mg, 0.052 mmol, 79%. $\epsilon_{786} = 2.3 \times 10^5$ M^{-1} cm^{-1}. The quantum yield in PBS was measured relative to IR125 in DMSO [$\phi = 0.13$, (2)] and found to be 0.07. ^1H NMR(CD_3OD) δ 1.42 (s, 12H), 1.47 (m, 4H), 1.68 (m, 4H), 1.85 (m, 4H), 2.10 (m, 2H), 2.30 (m, 4H), 2.85 (m, 4H), 3.97 (s, 8H), 4.23 (m, 4H), 6.40 (d, J = 13.9 Hz, 2H), 6.94 (d, J = 13.8 Hz, 2H), 7.3–7.5 (m, 8H), 7.55 (d, J = 7.7 Hz, 2H), 8.36 (d, J = 7.7 Hz, 2H).

BHMI

To a solution of BHCl (10 mg, 0.013 mmol) in DMF (0.2 mL) was added 1-methylimidazole (30 mg, 0.37 mmol). After 5 days, TLC analysis on reverse-phase with methanol as eluant showed no remaining starting material. Solvent was evaporated and the residue dissolved in methylene chloride (0.15 mL) and precipitated with hexane (1 mL). The solution and precipitation was repeated to provide a dark oil. Yield: 7 mg, 0.008 mmol, 80%. ^1H NMR(CD_3OD) δ 1.45 (s, 12H), 1.48 (m, 4H), 1.68 (m, 4H), 1.85 (m, 4H), 2.1 (m, 2H), 2.35 (m, 4H), 2.85 (m, 4H), 4.22 (m, 4H), 4.25 (s, 3H), 6.4 (d, J = 14 Hz, 2H), 6.88 (d, J = 14 Hz, 2H), 7.3–7.5 (m, 8H), 7.52 (br s, 1H), 7.57 (br s, 1H). The proton at the 2-position of the imidazolium moiety was not found.

4,5-Benzo-1-(5′-carboxypentyl)-2,3,3-trimethylindolinum bromide

A mixture of 4,5-benzo-2,3,3-trimethylindoline (0.5 g, 2.4 mmol) and bromohexanoic acid (0.5 g, 2.6 mmol) was stirred under nitrogen at 110°C for 2 h. The black tar was triturated 20 mL acetone. The gray powder was filtered and air dried. Yield: 0.8 g, 2 mmol, 83%. ^1H NMR (CD_3OD) δ 1.59 (m, 2H), 1.73 (m, 2H), 1.85 (s, 6H), 2.06 (m, 2H), 2.35 (t, J = 7.0 Hz, 2H), 4.64 (t, J = 7.7 Hz, 2H), 7.7–8.5 (m, 6H). The 2-methyl protons are acidic and exchanged with the deuterated solvent.

2-[4′-Chloro-7′-(4″,5″-benzo-1″-[5‴-carboxypentyl]-3″,3″-dimethylindolin-2″-ylidene)-3′,5′-(propane-1⁗,3⁗-diyl)-1′,3′,5′-heptatrien-1′-yl]-4,5-benzo-1-(5′′′′′-carboxypentyl)-3,3-dimethylindolinium bromide (NHCl)

A mixture of 4,5-benzo-1-(5′-carboxypentyl)-2,3,3-trimethylindolinum bromide (110 mg, 0.28 mmol), *N*-[5-anilino-3-chloro-2,4-propane-1′,3′-diyl)-2,4-pentadien-1-ylidene]anilininum chloride (50 mg, 0.14 mmol) and sodium acetate (100 mg, 0.72 mmol) was dissolved in ethanol (20 mL). After stirring at room temperature for 15 h under N_2, the solvent was removed under reduced pressure. The residue was triturated with a 1:1 solution of ethyl acetate and hexane (100 ml) to give a dark green solid. The solid was washed with 1 N HCl (5 mL) and dried in vacuo. Yield: 68 mg, 0.79 mmol, 56%. ^1H NMR(CD_3OD): δ 1.56 (m, 4H), 1.73 (m, 4H), 1.94 (m, 4H), 1.98 (m, 2H), 2.04 (s, 12H), 2.34 (m, 4H), 2.75 (m, 4H), 4.3 (m, 4H), 7.5 (m, 2H), 7.65–8.26 (m, 12H), 8.56 (m, 2H).

NHMI

A solution of NHCl (10 mg, 0.012 mmol) and *N*-methylimidazole (20 mg, 0.25 mmol) in 50 μl of DMF was allowed to stand for 7 days. The solvent was then removed under reduced pressure and the residue was dissolved in 200 μl of methylene chloride and 1 ml of ethyl acetate was added to precipitate the product. Yield: 3.2 mg, 0.0034 mmol, 28%. ^1H NMR (CD_3OD) δ 1.42 (m, 4H), 1.67 (m, 4H), 1.75 (s, 12H), 1.90 (m, 4H), 2.15 (m, 2H), 2.25 (m, 4H), 2.80 (m, 4H), 4.2 (m, 4H), 4.35 (s, 3H), 6.25 (d, J – 14 Hz, 2H), 6.88 (d, J = 14 Hz, 2H), 7.45 (d, J = 8.8 Hz, 2H), 7.55 (m, 2H), 7.65 (m, 2H), 7.83 (d, J = 1.8 Hz, 1H), 7.95 (m, 4H), 8.12 (d, J = 8.5 Hz, 2H), 8.28 (d, J = 1.8 Hz, 1H). The proton at the 2-position of the imidazolium moiety was not found.

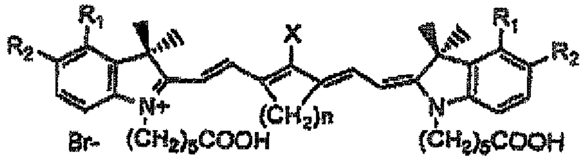
BPMP

A solution of 1-(5′-carboxypentyl)-2,3,3-trimethylindolinium bromide (100 mg, 0.28 mmol), *N*-[5-anilino-3-chloro-2,4-(ethane-1′,3′-diyl)-2,4-pentadien-1-ylidene]anilinium chloride (50 mg, 0.15 mmol), 4-morpholinopyridine (92 mg, 0.56 mmol) and acetonitrile (2 mL) was refluxed for 3 min. The solution was concentrated to dryness and triturated with refluxing ethyl acetate (2 × 20 mL). The dark green solid was purified by chromatography in two portions on octadecyl-functionalized silica gel (5/8″ × 3″). The column was eluted with a step-gradient of acetonitrile and 0.1 % trifluoroacetic acid: 20% acetonitrile (40 mL), 30% acetonitrile (60 mL), and 40% acetonitrile (40 mL). Fractions containing the dye were concentrated to dryness and triturated with ether (2 mL). Yield: 13 mg, 0.015 mmol, 11%. ^1H NMR (CD_3OD) δ 1.5 (m, 4H), 1.53 (s, 12H), 1.7 (m, 4H), 1.9 (m, 4H), 2.32 (t, J = 7 Hz, 4H), 3.14 (s, 4H), 3.93 (s, 8H), 4.2 (t, J = 7 Hz, 4H), 6.22 (d, J = 14 Hz, 2H), 7.22 (d, J = 14 Hz, 2H), 7.3–7.5 (m, 10H), 8.55 (d, J = 8 Hz, 2H).

Dye-Succinimidyl Esters

A general method for preparing succinimidyl esters of the dialkylpyridinium cyanine dyes is described, using the specific example of BHDMAP. To a solution of BHDMAP (2 mg, 2 mmol) in DMF was added *N*-hydroxysuccinimide (3 μg, 19 mmol) and dicyclohexylcarbodiimide (2 mg, 10 μmol). The reaction progress was monitored by HPLC on a Poros R2 column (20% to 40% acetonitrile vs. 0.1 M triethylammonium acetate over three min, 5 mL/

Table 1
Properties of Heptamethine Dyes



Dye	Substituents	Abs max in PBS (nm)	Em max in PBS (nm)	Aggregates in high salt?	Half-life in PBS/ azide/EDTA
BHCI	$R_1 = R_2 = H$ $X = Cl$ $n = 3$	780	—	yes	—
BHDMAP	$R_1 = R_2 = H$ X = 4-dimethylaminopyridinium chloride $n = 3$	786	801	no	>6 mon
BHMP	$R_1 = R_2 = H$ X = 4-morpholinopyridinium chloride $n = 3$	786	801	no	>6 mon
BHMI	$R_1 = R_2 = H$ X = 3-methylimidazolium chloride $n = 3$	792	805	no	<1 mon
BPMP	$R_1 = R_2 = H$ X = 4-morpholinopyridinium chloride $n = 2$	816	843	no	3 days
NHMI	R_1, R_2 = -CH=CH-CH=CH- X = 3-methylimidazolium chloride $n = 3$	831	855	yes	—

min). After 6 h the reaction mixture contained starting dye (17%), monoester (60%) and diester (23%). Acetic acid (20 µL) and methanol (0.5 mL) were added and the solution filtered to remove dicyclohexylurea. The solution was concentrated to dryness and redissolved in DMF (0.5 mL). The concentration of the succinimidyl ester solution was determined by dilution of an aliquot into phosphate buffered saline and measurement of the optical density at 786 nm. The concentration of BHDMAP succinimidyl ester was found to be 12 mg/mL.

Absorbance Spectra of Dyes

A stock solution of dye in DMF or acetonitrile was diluted into low (0.1 M NaCl, 50 mM phosphate, pH 7) and high (3.8 M NaCl, 50 mM phosphate, pH 7) salt solutions at a concentration of 1×10^{-5} M. The absorbance of each solution was measured from 500–900 nm. For determination of aqueous stability, solutions of dye in phosphate buffered saline (10 mM phosphate, 120 mM NaCl, 2.7 mM KCl, pH 7.4) with sodium azide (6 mM) and EDTA (6 mM) were periodically monitored at their absorbance maxima.

Photodecomposition of Dyes

Solutions of dyes in phosphate buffered saline were irradiated in 1-mL methylacrylate disposable cuvettes with a halogen lamp. The light was directed to the cuvette via an optical fiber. The initial absorbance of each dye varied from 0.5 to 1.7 O.D. units. The absorbance of the dye solutions at the absorbance maximum of each dye was monitored periodically. From a plot of the logarithm of the normalized absorbance ($\ln [\text{Dye}]/[\text{Dye}]_0$) vs. time, the half-life of each dye was determined from least-squares analysis.

Antibody Labeling

A general method for labeling antibodies is described, using the specific examples of αCD4 and BHDMAP succinimidyl ester. To a solution of Anti-Leu-3a (αCD4, 100 µL, 2.5 mg/mL) was added BHDMAP succinimidyl ester (1.3 µL, 12 mg/mL) and bicarbonate buffer (5 µL, 1 M, pH 9.2). After 15 min at ambient temperature the excess dye was removed by size exclusion gel filtration. The dye/protein ratio was determined by UV-visible absorbance spectra. The extinction coefficient of the protein was assumed to be 170,000 cm^{-1} M^{-1} at 280 nm.

Cell Labeling

A mixture of three dye-labeled antibodies in PBS/BSA solution was prepared: Cy5-αCD3 (150 µg/mL), Cy5.5-αCD4 (100 µg/mL), and BHMP-αCD8 (150 µg/mL). Blood from in-house donors (90 µL), dye-labeled antibodies (10 µL) and detergent solution (3 µL) were mixed in an eppendorf tube and allowed to incubate at ambient temperature for 20 min. The mixture was drawn into a 35 µm rectangular capillary and scanned.

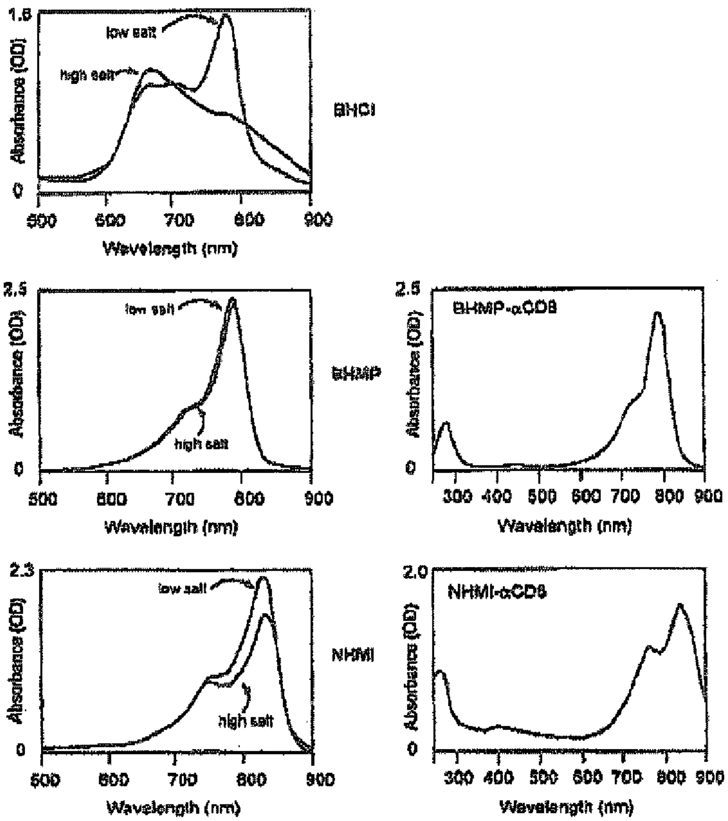


FIG. 1. Absorbance spectra of dyes in two salt solutions (0.1 M and 3.8 M NaCl) and conjugated to antibody.

Volumetric Capillary Cytometry

Two lasers, a 3-mW He-Ne at 633 nm and a 50-mW diode laser at 785 nm, were used as excitation sources. The power from the He-Ne laser on the sample was 1 mW. The diode laser was operated at 30 mW, and the power from the diode laser on the sample was 10 mW. A combination of cylindrical microlenses and molded glass aspheres compensated the diode laser astigmatism and provided a collimated, circular output beam with a diameter of 1 mm (18). After collimation, a line pass filter removed broad band spontaneous light emitted from the lasers. The two lasers were aligned using a dichroic beam combining mirror so that the laser beams overlapped and were parallel to approximately 0.1 mrad. The combined laser beams were directed to a scanning mirror mounted on a precision galvanometer with approximately 6 degrees of scan range. After reflection off the scan mirror the beams passed through a series of transfer lenses and were focused on the capillary to a diameter of 10 μm with a 20×, 0.45 NA microscope objective. The 6 degree scan range resulted in a 1 mm transverse scan length across the width of the capillary in the focal plane of the objective. The capillary holder rested on a precision translation stage driven by a stepper motor that provided a longitudinal translation down the long axis of the capillary.

The fluorescent emission from the capillary was collected by the microscope objective and retraced the beam path of the laser through the lens relay system and scan mirror. A series of line filters provided approximately 10^{-8} attenuation of the laser emission while passing 50% of the dye fluorescence signal. The fluorescence signal was focused by a 160 mm lens onto a 1 mm aperture to further discriminate against direct reflections of the laser beam from surfaces outside of the focal plane of the scan objective. The different spectral regions were separated using one or more dichroic mirrors. The photomultipliers tubes (PMTs) were chosen to have high gain, good quantum efficiency, and extended response in the IR spectral region. The outputs from the PMTs were sent to a transimpedance amplifier and an active filter that limited the signal bandwidth to approximately 1 kHz.

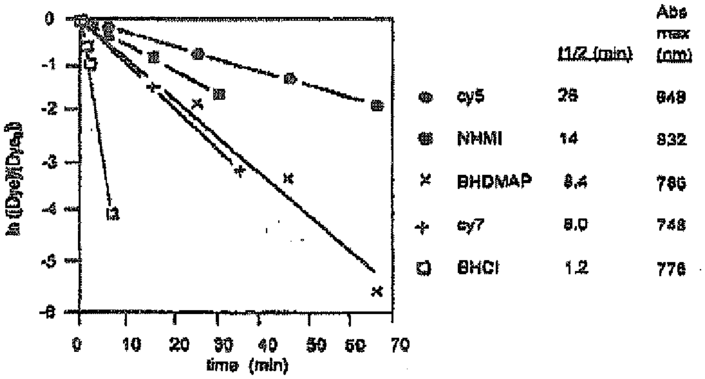


FIG. 2. Comparison of the photostability of cyanine dyes. Dyes dissolved in phosphate buffered saline solution in methylacrylate cuvetres were irradiated with a halogen lamp. The concentrations were monitored by absorbance. Half-lives and absorbance maxima are given for each dye.

The filtered signals were digitized by a high speed, 16 bit A/D converter and stored in the memory of a laboratory computer.

RESULTS

Dyes

Several pyridinium and imidazolium dyes were synthesized and characterized by ^1H NMR, absorbance and fluorescence (Table 1). The dyes were initially evaluated for their tendency to aggregate on proteins. Aggregation can be simulated by measuring the absorbance of the dye in low and high salt solutions. High salt solutions perhaps simulate the environment of the dye in high local concentration on the surface of a protein. The spectra of several dyes in low (0.1 M NaCl, 50 mM phosphate, pH 7) and high (3.8 M NaCl, 50 mM phosphate, pH 7) salt solutions, and covalently attached to antibodies were measured (Fig. 1). BHCl appears to aggregate even in low salt, and in high salt the absorbance maximum shifts to shorter wavelength. The more ionic dye, BHMP, shows little aggregation in low and high salt solutions. Upon covalent attachment to antibody, the absorbance maximum of BHMP succinimidyl ester shifts by 7 nm to longer wavelength (from 788 to 795 nm) with a twofold decrease in fluorescence. Very little of the aggregated form is present, as noted by the very slight increase of the "shoulder" in the absorbance spectrum at 740 nm.

The longest wavelength dye, NHMI, shows more significant stacking in both high salt and on the protein surface. In high salt solution, the aggregated form appears as a peak at 770 nm in the absorbance spectrum. Upon antibody labeling, a 17 nm shift to longer wavelength (from 831 to 848 nm) and a fourfold decrease in fluorescence is observed. Appearance of the aggregated form is noted in the absorbance spectrum.

The pyridinium dyes, BHDMAP and BHMP, are indistinguishable in their absorbance, fluorescence and stability properties, and are used interchangeably. Both are stable at pH 7 for at least several months, and withstand labeling conditions at pH 9.0. Substituting the cyclohexene group with cyclopentene (BPMP) or substituting the pyridinium with imidazolium (BHMI) produces dyes that are considerably less stable. BHDMAP and BHMP rapidly decompose at pH 10 and would not be suitable for labeling materials that are utilized in alkaline conditions.

The relative photostabilities of several cyanine dyes were measured in phosphate buffered saline (Fig. 2). The most stable dye is the pentamethine dye, Cy5. The remaining dyes are heptamethine dyes, of which the least stable dye is the chloro-substituted dye, BHCl. Substitution of the chloride with dimethylaminopyridine to provide BHDMAP improves the photostability in water sevenfold. The photostabilities of the pyridinium and imidazolium dyes, BHDMAP and NHMI, are comparable to the photostability of the arylsulfonate heptamethine dye, Cy7.

CELL ANALYSIS

A three-color volumetric capillary cytometry (VCC) laser scanner device was constructed to utilize the dyes Cy5, Cy5.5 and BHMP (Fig. 3). The laser scanner performs a raster scan of a precision rectangular capillary. Fluorescence emission from the capillary is separated into wavelength intervals by dichroic mirrors, and the emission intensity in these spectral regions is measured with three photomultipliers. The intensity information is stored in a computer and, after completion of the scan, this information is used to reconstruct an image of the capillary.

Cell enumeration of CD3+CD4+ and CD3+CD8+ populations is performed in one capillary using Cy5-αCD3, Cy5.5-αCD4 and BHMP-αCD8 (channels 0, 1 and 2, respectively). Incubation and scanning are performed in undiluted, whole blood. Potential cells of interest are identified by analyzing the Cy5-αCD3 fluorescence image plane. A threshold fluorescence level for cell detection is

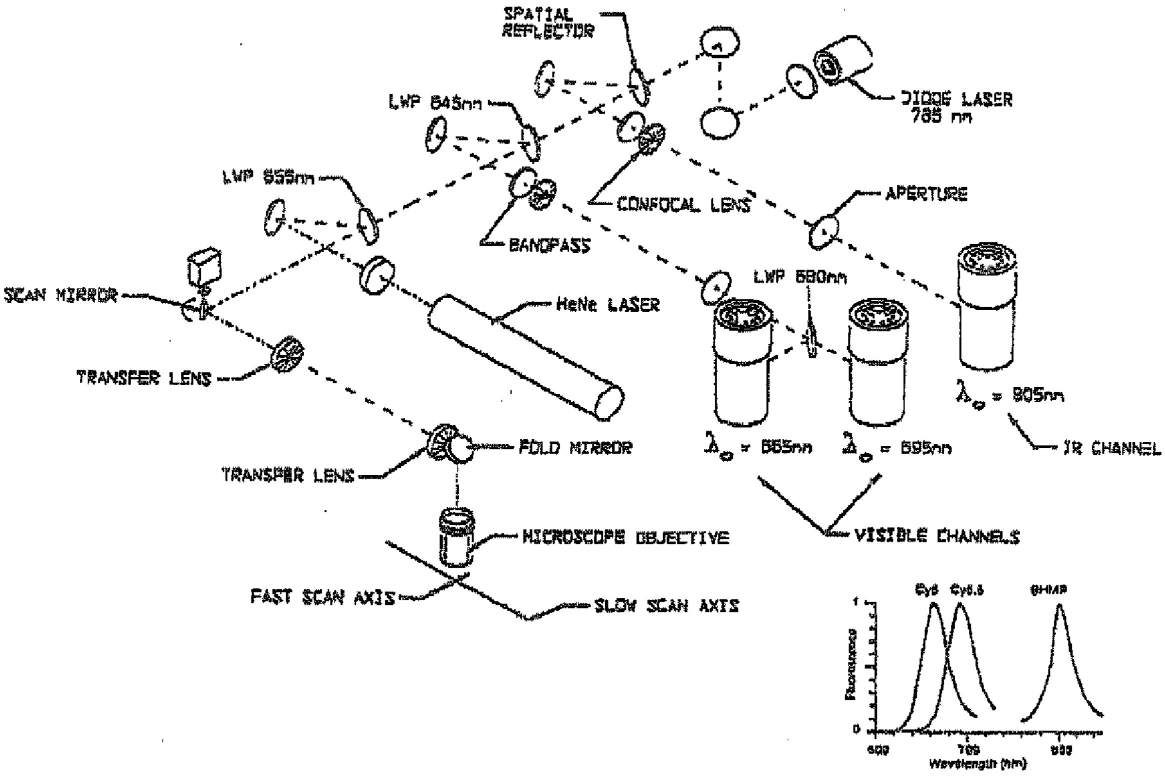


FIG. 5. Schematic diagram of a two-laser, three-color volumetric capillary cytometer. The inset shows normalized emission spectra of the three fluorescent dyes.

set at a level just above the background fluorescence from free dye-labeled antibody. The location of the center of the cell is determined by finding the pixel position with the greatest intensity in the vicinity of a potential cell. The cell center location in the Cy5-αCD3 image is used as a starting point to find matching, nearby maximum fluorescence signal locations in the other image planes. These maximum fluorescence values are stored, representing measurements of the corresponding antigen densities on the cell surface. Plots of each of the remaining fluorescence channels vs. the Cy5-αCD3 channel provides dot plots of CD3+CD4+ and CD3+CD8+ populations (Fig. 4). Cell counts were similar (within 10%) to results from two separate reactions on a He-Ne system (Cy5-αCD3/Cy5.5-αCD4 and Cy5-αCD3/Cy5.5-αCD8).

DISCUSSION

Substitution reactions on cyanine dyes are well known. Preceding the aromatic thiol substitution of heptamethines described by Strekowski et al. (19, 20), substitution of *meso*-alkoxy and -alkylthio groups of trimethincyanines with amines was described in British patents (5). *Meso*-substituted trimethines were also provided by the addition of 1,3-diethyl-2-thiobarbituric acid to acetylenic dyes (12). Substitution products of 4-dimethylaminopyridine (DMAP) and 1-methylimidazole with acid anhydrides are generally fleeting intermediates in ester syntheses (7). Stable addition products of these reagents to a variety of electrophiles have been characterized, some that yield fluorescent products (1,4). However, the addition product of dialkylaminopyridines and cyanine dyes to provide ionic dyes has not been previously described.

The pyridinium-substituted dyes, BHDMAP and BHMP, are effective dyes for cell analysis. They are simple to synthesize and activate, and exhibit little aggregation on covalent attachment to proteins. Their absorbance matches the readily available diode laser at 785 nm and therefore provides an inexpensive color in cellular analysis. Their disadvantages include lower photostability than Cy5, instability to alkali, and relatively low quantum yields.

The goal of a second IR color was not achieved for several reasons. The two longer wavelength dyes, BPMP

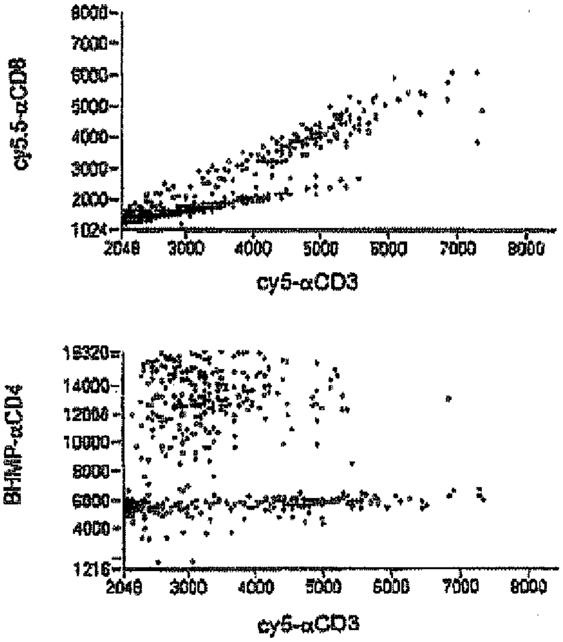


FIG. 4. Three-color volumetric capillary cytometry. Whole blood was incubated with three dye-labeled antibodies: Cy5-αCD3, Cy5.5-αCD8, BHMP-αCD4. Uncompensated dot plots are shown. Cy5-αCD3 was used to identify cellular events.

and NHMI, are unsuitable: BPMP is not stable in water, and NHMI exhibits aggregation, and therefore lowered fluorescence, when conjugated to antibody. In addition, photomultipliers that are sensitive in the region >840 nm are not routinely available.

Cell enumeration using dye-labeled antibodies to CD3, CD4 and CD8 is commonly performed by flow cytometry. A commercially available protocol (Becton Dickinson) utilizes two-color labeling and requires two separate antibody reactions to count both CD3+CD4+ and CD3+CD8+ populations. The advantages of three-color analysis are several: fewer antibody reactions are required for multiparameter reactions, and certain subpopulations that can only be distinguished by three parameters can be analyzed. A disadvantage is the requirement for more complex data analysis.

In our scanning instrument, the signal intensities of Cy5 and BHMP are approximately matched. Although the quantum yield of BHMP ($\phi = 0.07$) is lower than Cy5 ($\phi = 0.20$), the laser power on the sample is higher for IR excitation (10 mW) than visible excitation (1 mW). Channel 0 (Cy5) and channel 2 (BHMP) are also completely orthogonal. The lack of overlap of the emission of the two dyes is reflected in the data; a horizontal line can be drawn separating the CD3+CD8− and CD3+CD8+ populations (Fig. 4). The two visible dyes, Cy5 and Cy5.5, overlap at 60% of their normalized emission spectra. The overlap is usually compensated by applying a correction based on instrument response to the pure dyes. After compensation, a horizontal line can also be drawn separating the CD3+CD4− and CD3+CD4+ populations. Our three-color system has the advantage of inexpensive and efficient lasers compared to three-color systems (e.g., fluorescein, PE, PE-Cy5) based on the argon ion laser. Disadvantages include the relatively large spectral overlap of the two visible dyes, and the added complexity of a dual-laser system. Future work will include a dual laser diode system that will allow higher laser powers and greater sensitivity than systems utilizing a He-Ne laser.

LITERATURE CITED

1. Adamiak RW, Biala E, Skalski B: New, ionic side-products in oligonucleotide synthesis: Formation and reactivity of fluorescent N-/purin-6-yl/pyridinium salts. Nucleic Acids Res 13: 2989–3003, 1985.
2. Benson RC, Kues HA: Absorption and fluorescence properties of cyanine dyes. J Chem Eng Data 22:379–383, 1977.
3. Dunn KW, Mayor S, Myers JN, Maxfield FR: Applications of ratio fluorescence microscopy in the study of cell physiology. FASEB J 8:573–582, 1994.
4. Eadie JS, Davidson DS: Guanine modification during chemical DNA synthesis. Nucleic Acids Res 15:8333–8349, 1987.
5. Hamer FM: The Cyanine Dyes and Related Compounds. John Wiley and Sons, New York, 1964.
6. Haugland RP: Handbook of Fluorescent Probes and Research Chemicals. Molecular Probes, Eugene, OR, 1992–1994.
7. Höfle G, Steglich W, Vorbrüggen H: 4-Dialkylaminopyridines as highly active acylation catalysts. Angew Chem Int Ed Engl 17:569–583, 1978.
8. Joos S, Fink TM, Rätsch A, Lichter P: Mapping and chromosome analysis: The potential of fluorescence in situ hybridization. J Biotechnol 35:135–153, 1994.
9. Lanier LL, Recktenwald DJ: Multicolor immunofluorescence and flow cytometry. Methods: A Companion to Methods Enzymol 2:192–199, 1991.
10. Lee K-B, Al-Hakim A, Loganathan D, Linhardt RJ: A new method for sequencing linear oligosaccharides on gels using charged, fluorescent conjugates. Carbohydr Res 214:155–168, 1991.
11. Manian BS, Dubrow B, Hartz T: Clinical evaluation of volumetric capillary cytometry—a new method for absolute cell counts in homogeneous format. Anal Cell Pathol 6:217–218, 1994.
12. Mee JD: A new resonance system. Ethynologous amidinium compounds. Acetylenic analogs of cyanine dyes. J Am Chem Soc 96: 4712–4714, 1974.
13. Middendorf LR, Bruce JC, Bruce RC, et al.: Continuous, on-line DNA sequencing using a versatile infrared laser scanner/electrophoresis apparatus. Electrophoresis 13:487–494, 1992.
14. Mujumdar RB, Ernst LA, Mujumdar SR, Lewis CJ, Waggoner AS: Cyanine dye labeling reagents: Sulfoindocyanine succinimidyl esters. Bioconjugate Chem 4:105–111, 1993.
15. Patel KM, Sparrow JT: Phase transfer catalysis: Reaction of 4-bromopyridine with cyclic secondary amines. Synth Comm 9:251–255, 1979.
16. Reynolds GA, Drexhage KH: Stable heptamethine pyrylium dyes that absorb in the infrared. J Org Chem 42:885–888, 1977.
17. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
18. Snyder JJ, Reichert P, Baer T: Fast diffraction-limited cylindrical microlenses. Appl Optics 30: 2743–2747, 1991.
19. Strekowski L, Lipowska M, Patonay G: Substitution reactions of a nucleofugal group in heptamethine cyanine dyes. Synthesis of an isothiocyanato derivative for labeling of proteins with a near-infrared chromophore. J Org Chem 57:4578–4580, 1992.

20. Strekowski L, Lipowska M, Patonay G: Facile derivatizations of heptamethine cyanine dyes. Synth Comm 22:2593–2598, 1992.
21. Tanke HJ, Van der Keur M: Selection of defined cell types by flow-cytometric cell sorting. Trends Biotechnol 11:55–62, 1993.
22. Terpetschnig E, Szmacinski H, Ozinskas A, Lakowicz JR: Synthesis of squaraine-N-hydroxysuccinimide esters and their biological application as long-wavelength fluorescent labels. Anal Biochem 217:197–204, 1994.
23. Trainor GL: DNA sequencing, automation and the human genome. Anal Chem 62:418–426, 1990.
24. Waggoner AS, Ernst LA, Mujumdar RB: Method for labeling and detecting materials employing arylsulfonate cyanine dyes. U.S. Patent 5,268,486, 1993.